HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2015 October 14.

Published in final edited form as:

Nat Immunol. 2015 September ; 16(9): 961–969. doi:10.1038/ni.3231.

T cell receptor dwell-times control the kinase activity of ZAP-70

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Abstract

Kinase recruitment to membrane receptors is essential for signal transduction. However, the underlying regulatory mechanisms are poorly understood. We investigated how conformational changes control T cell receptor (TCR) association and activity of ZAP-70 kinase. Structural analysis of ZAP-70 showed that TCR binding or phosphorylation trigger transition from the closed/auto-inhibited conformation to an open conformation. Using ZAP-70 mutants with defined conformations, we found that TCR dwell-times control kinase activity. The auto-inhibited conformation minimizes receptor dwell-times and thereby avoids activation by membrane-associated kinases. Parallel recruitment of co-receptor-associated Lck kinase to the TCR ensures ZAP-70 phosphorylation and stabilizes ZAP-70 binding. Our study suggests that recruitment dynamics of cytosolic enzymes to the membrane regulate the activity and function of receptors lacking intrinsic catalytic activity.

Introduction

Plasma membrane signaling often requires recruitment of cytosolic enzymes including kinases, phosphatases and hydrolases. Most pathways utilize inducible binding to membrane proteins by exposing or creating interaction motifs through conformational changes or post-translational modifications. Cytosolic enzymes frequently assume auto-inhibited conformations and are only active upon receptor binding. Despite their universal presence, possible regulatory functions of assembly dynamics have been overlooked. This is most likely because previous approaches have been limited by the speed of individual events and a lack of structural information. We choose T cell activation as a model system to reveal that conformation-dependent receptor interaction dynamics control catalytic activities. The ζ-associated protein of 70 kDa (ZAP-70) is a typical example of an inactive cytosolic tyrosine kinase that is recruited to a transmembrane receptor lacking intrinsic catalytic activity[1, 2, 3, 4, 5, 6, 7]. ZAP-70 and the related spleen tyrosine kinase (Syk)[8] are central to all cellular immune responses. They associate with numerous surface receptors including the T cell, B cell, Fc and integrin receptors. T cell receptor (TCR) signaling is initiated by recognition of peptide presenting major histocompatibility complexes (pMHC) on antigen-

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presenting cells (APCs)[9] (Fig. 1a). The leukocyte-specific protein tyrosine kinase (Lck) is recruited to the TCR via its association with the co-receptors CD4 or CD8, which also bind pMHC[10]. Lck is activated by trans-autophosphorylation and it, in turn, phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR/CD3 complex[11]. ZAP-70 is recruited to the doubly phosphorylated ITAMs (pITAMs) via its Src homology 2 (SH2) domains[12]. CD3-bound ZAP-70 is activated by both Lck and (trans)-autophosphorylation[13, 14]. ZAP-70 then phosphorylates its downstream substrates including the linker for activation of T cells (LAT)[15].

ZAP-70 (Fig. 1b) contains two SH2 domains connected via interdomain-A (I-A), commonly referred to as the tandem SH2 domain module (tSH2)[6, 7, 16, 17]. The flexible interdomain-B (I-B) connects the tSH2 and the kinase domain (KinD). Previous structural analyses show that tyrosines Y315 and Y319 in I-B cause a closed/auto-inhibited conformation by binding specific pockets within I-A and the KinD, respectively. This auto-inhibited conformation has been thought to render the kinase catalytically inactive. TCR binding is hypothesized to loosen the tSH2-KinD interaction[17], thus facilitating phosphorylation of Y315/Y319 by either Lck[14, 18] or (trans)-autophosphorylation[13]. Mutation of Y315/Y319 to phenylalanines or alanines prevents appropriate T cell activation[13, 14, 17, 18, 19, 20, 21, 22, 23]. Phosphorylating Y492 and Y493 in the activation loop of the KinD by either Lck[24, 25, 26] or by trans-autophosphorylation[18] controls the catalytic activity of TCR-bound ZAP-70.

We obtained structural information for pITAM-associated and/or Y315/Y319 phosphorylated ZAP-70 by Hydrogen-Deuterium Exchange (HDX) – Mass Spectrometry (MS)[27]. Our data show that receptor binding and/or phosphorylation induce an open conformation. The phenylalanine mutant Y315F/Y319F prefers the closed/auto-inhibited conformation and opens only upon receptor binding. In contrast, the alanine mutant Y315A/Y319A is always in an open conformation. We utilized these mutants to show that different ZAP-70 conformations (‘closed’ or ‘open’) control TCR binding kinetics, but not its intrinsic catalytic activity. Due to very short receptor dwell-times, ZAP-70 phosphorylation requires co-recruitment of Lck. Phosphorylation of Y315/Y319 stabilizes ZAP-70 binding and facilitates its full phosphorylation/activation. Our findings suggest that separating receptors from their catalytic activity is well suited for pathways that require efficient silencing in resting cells as well as fast and strong responses upon ligand binding. In these pathways, the binding dynamics themselves regulate activation.

Results

HDX–MS reveals conformational changes in ZAP-70

We obtained structural insights into the kinase-dead D461N mutant of ZAP-70 (YY_{DN}) by HDX–MS. In brief, deuterium in solution (D_2O) replaces amide hydrogens in ZAP-70′s backbone. Mass spectrometry of subsequent pepsin digests allows quantification and assignment of HDX to specific amino acid (aa) sequences. HDX rates are influenced by solvent exposure and hydrogen bonds. Changes in HDX patterns locate conformational changes, intra- and intermolecular interaction sites induced by complex formation and/or post-translational modifications (Fig. 1c–f). Thus, HDX-MS provides structural insights on different activation states of soluble ZAP-70 that have been unattainable to date. However,

HDX–MS does not generate an actual structure and has lower resolution than crystallography. Conformational changes upon pITAM-binding were identified by comparison of HDX between YY_{DN} alone (used as reference standard for all HDX–MS measurements in this study) and in complex with the phosphorylated cytosolic domain of the TCR γ-chain, pCD3γ_{CD} (YY_{DN}^{cplx}). Differences in HDX were projected onto the known auto-inhibited structure of YY_{DN} (Fig. 2a)[17] and compared by alignment (Supplementary Fig. 1). Reduced HDX reveals regions of interactions between the pCD3γ_{CD} and theZAP-70 SH2 domains (aa E174-F187, I203-L212 and L239-L250). Increased HDX in I-A (aa D116-L152), I-B (aa K251-L287) and the KinD (aa Y598-L600) indicates separation of tSH2 and KinD upon pCD3γ_{CD} binding (illustrated in Supplementary Fig. 2a). The binding pocket for Y319 in the N-terminal (N)-lobe of the KinD shows no changes, suggesting that this interaction between I-B and the KinD is maintained upon pITAM-binding. Additional hydrogen bond rearrangements were observed in the KinD through reduced HDX in its N-lobe (aa G350-V356; I368-M383) and C-terminal (C)-lobe (aa L469; Y535-L568). These data show that pITAM binding alone is sufficient to induce the open conformation of ZAP-70 and rearrangement of the KinD.

Based on the auto-inhibited structure, phosphorylation of Y315/Y319 has been proposed to induce an open conformation and consequently catalytic activity of ZAP-70[16, 17]. All recombinant ZAP-70s were analyzed for phosphorylation of Y292, Y319, Y492, Y493 and total tyrosine-phosphorylation by immunoblotting to confirm phosphorylation of Lck-treated proteins (Supplementary Fig 3). Furthermore, no non-phosphorylated peptides containing Y292, Y315, Y319, Y492 and Y493 were detected by MS analyses after ZAP-70 phosphorylation. Phosphorylated ZAP-70 (pYY_{DN}) showed increased HDX in I-A (aa D116-L152) and I-B (aa Y315-L332) (Fig. 2a and Supplementary Fig. 1). These data indicate that phosphorylation of Y315/Y319 separates tSH2 and the KinD (illustrated in Supplementary Fig. 2b). Additional HDX increases in I-B (aa Y315-L332) and the N-lobe of the KinD (aa R359-D365) indicate dissociation of Y319 from its binding pocket. The phenylalanine mutant Y315/Y319F (FF_{DN}) was analyzed to exclude the possibility that phosphorylation outside of I-B induces the open conformation (Fig. 2b and Supplementary Fig. 1). HDX for non-phosphorylated FF_{DN} is reduced in I-A (aa D116-L118; Y126-G135; E139-L152), I-B (N288-T312) and the KinD (aa M384-Q388; T494-C510; E531-L568; Y598-L600). HDX–MS also detects shifts in conformational equilibria[28] and these reductions indicate ZAP-70 oscillates between closed/auto-inhibited and open conformations. FF_{DN} is more prone to the former, due to the destabilizing effects of the hydroxyl groups on the binding of Y315 and Y319 to I-A and the KinD, respectively. Phosphorylated FF_{DN} (pFF_{DN}) showed only minor HDX differences at the beginning of I-B (K251-A267). This confirmed that Y315/Y319 phosphorylation is essential for the release of Y319 from its binding pocket and ZAP-70 assuming an open conformation without pITAM binding. Next we tested if FF_{DN} in complex with pCD3γ_{CD} (FF_{DN}^{cplx}) is still able to assume an open conformation. HDX was increased in I-A (E136-A137; I142-L152) and I-B (K251-A267; N288-T312), suggesting that FF_{DN}^{cplx} changes into an open conformation upon receptor binding similar to YY_{DN}^{cplx}. These data show that opening of ZAP-70 through pITAM-binding and phosphorylation are independent processes with similar outcomes. To test for combinatorial effects, we analyzed phosphorylated YY_{DN} and phosphorylated FF_{DN}

in complex with pCD3γ_{CD} (pYY_{DN}^{cplx} and pFF_{DN}^{cplx}) (Supplementary Figs. 1 and 4). HDX–MS shows that the effects of pITAM-binding and phosphorylation are additive.

Previous reports show that the alanine mutant Y315/Y319A (AA) has reduced activity in T cells, but increased activity in TCR-independent activation systems[17, 18, 23]. HDX in I-A and I-B of AA_{DN} are the highest observed (Fig. 2c and Supplementary Fig. 1) and HDX in I-B and KinD include regions previously not detected (aa K333-L338; I342-M359; I368-E382; R385-Q388; L412-M414; S483-Y492; A601-G606). AA_{DN} binding to pCD3γ_{CD} and/or phosphorylation of AA_{DN} by Lck slightly revert the effects of the alanine mutations (Fig. 2c and Supplementary Fig. 1 and 4). However, for all forms of AA_{DN}, the Y319 binding pocket is unoccupied and no interaction between I-B and KinD can be formed. These data suggest that the phenyl groups of Y315/Y319 are required to assume an auto-inhibited and structurally stable conformation. Consequently, AA_{DN} is constitutively in an open conformation with the tSH2 and KinD separated.

We tested if the tSH2–KinD interaction is only loosened[17] or completely disrupted by pITAM-binding or ZAP-70 phosphorylation. A dissociated tSH2 would have the same solvent accessibility and therefore, the same HDX patterns as a recombinant tSH2 module (aa 1–256). Therefore, we compared HDX for pCD3γ_{CD}-bound and/or phosphorylated tSH2 with the corresponding forms of YY_{DN}, FF_{DN} and AA_{DN} (Fig. 2d and Supplementary Fig. 1 and 4). Comparable HDX increases and patterns are consistent with complete dissociation of tSH2 and the KinD. In addition, an expected effect of the open conformation is an increased volume. Such an effect is demonstrated by reduced retention times in size exclusion chromatography, which indicate increased diameters for the ZAP-70 forms with increased HDX in I-A and I-B (Fig. 3a–c).

While all pITAMs of the TCR bind ZAP-70 with similar binding affinities[29, 30, 31, 32], it is possible that the conformational changes seen here in response to pCD3γ_{CD} binding might be specific to the CD3γ subunit. Therefore, HDX–MS was performed on YY_{DN} bound to six phosphorylated peptides comprising the sequences of all TCR ITAMs (i.e. CD3γ, δ, ε, ζ1, ζ2 and ζ3). Increased HDX in I-A was observed in all complexes, demonstrating that TCR pITAMs cause ZAP-70 to open via a general mechanism (Fig. 3d). These data are supported by the reduced retention times of all ZAP-70–pITAM complexes in analytical size exclusion chromatography (Fig. 3e). Taken together, the HDX–MS data shows opening of ZAP-70 after pITAM-binding, phosphorylation or mutation of Y315/Y319 to alanine. We established that the phenylalanine mutant is preferentially in the closed conformation, while the alanine mutant is always open. Analyses of these mutants enable us to determine how distinct ZAP-70 conformations control T cell activation.

Conformation does not affect ZAP-70 catalytic activity

Multiple reports have shown effects of Y315/Y319 mutations on TCR signal transduction[17, 18, 23]. Wildtype (YY) and alanine (AA) mutants transduce TCR signals and trigger T cell activation; AA has increased background activity, but reduced activity upon TCR ligation. In TCR-independent activation systems AA shows higher activity than wildtype ZAP-70 (YY). The phenylalanine mutant (FF) cannot transduce signal or activate T cells. We activated ZAP-70 negative Jurkat T cells (P116) stably expressing ZAP-70–GFP

fusion proteins (^{GFP}YY, ^{GFP}FF and ^{GFP}AA) with increasing concentrations of stimulatory antibodies (Fig. 4a). At high antibody concentration ^{GFP}YY showed phosphorylation on Y493 (pY493) in the activation loop. In contrast, ^{GFP}AA and ^{GFP}FF showed little or no increase in pY493. Only ^{GFP}YY- and ^{GFP}AA-expressing P116 cells showed LAT phosphorylation on Y191 (pY191). ^{GFP}AA requires >4-fold more antibody than ^{GFP}YY to achieve similar LAT phosphorylation. These data confirm the published studies and further suggest that the reduced signal transduction of the Y315/Y319 mutants is due to lower numbers of activated kinase molecules and not to reduced catalytic activity.

Catalytic activity of ZAP-70 is reported to require the open conformation[16, 17]. We quantified both the potential of ZAP-70 to be activated by Lck and its catalytic activity towards its natural substrate, LAT, in a two-step *in vitro* kinase assay. Increasing amounts of Lck phosphorylated Y493 similarly in recombinant wildtype and mutant ZAP-70s (Fig. 4b). None of the kinases had catalytic activity without initial phosphorylation (Fig. 4c). In contrast, prior studies reported catalytic activity for purified or over-expressed AA in the absence of Lck[17, 18, 33]. We found that this activity is due to background phosphorylation/activity that becomes amplified upon addition of ATP. Evidently, the open conformation of ZAP-70 is more susceptible to non-specific phosphorylation during production/purification. To remedy this problem, YY and AA were expressed fused to the phosphatase domain of SHP-1 (which was removed during purification) to eliminate any background phosphorylation (Supplementary Fig. 5); this process yielded completely inactive ZAP-70s. Therefore, the open conformation (AA) is not sufficient to induce kinase activity or auto-phosphorylation. All forms of ZAP-70 had similar capacities for activation by Lck. While YY activity increased slightly faster, all forms of ZAP-70 reached comparable activities. Increasing ZAP-70 concentrations (70nM-1.4μM) had no effect on basal or relative activities, and only accelerated the kinetics of LAT phosphorylation. According to these data, all fully phosphorylated forms of ZAP-70 have equivalent molar activities. Our HDX–MS data shows that pFF_{DN}, despite being phosphorylated on Y493, is in the closed conformation. This suggests that both the closed/auto-inhibited and open conformations of ZAP-70 have similar potential to become activated as well as comparable catalytic activity towards their substrates. Based on these data, the cellular signaling defects of FF and AA cannot be explained through differences in their catalytic activity.

ZAP-70 conformations regulate TCR binding dynamics

Affects of ZAP-70 conformations on pITAM-binding capacities were tested in ELISA based competition assays (Fig. 5a). We found that FF requires ~3-fold higher concentrations than YY to compete similarly for $pCD3\gamma_{CD}$ binding (Fig. 5b and Supplementary Table 1). In contrast, AA and tSH2 can easily compete with >3 fold excess YY. These data suggest that the closed form of ZAP-70 (FF_{DN}) binds pITAMs less efficiently than the wildtype, while the open form (AA_{DN}) and tSH2 have increased pITAM-binding capacities.

Bio-layer interferometry (BLI) was used to determine dissociation constants (K_D) as well as on- and off-rates (k_{on} and k_{off}) for ZAP-70 binding to $pCD3\gamma_{CD}$ (Fig. 6 and Supplementary Table 2)[34]. The binding curves for YY_{DN} and FF_{DN} required fitting to a 2:1 heterogeneous ligand-binding model yielding two sets of binding constants. This suggests that they exist in

equilibria of two free- and two pITAM-bound conformations (Fig. 6b and Supplementary Table 2). AA_{DN} and tSH2 binding data fit a 1:1 binding model with one set of constants. All ZAP-70 had K_D1's of ~10nM. The corresponding $k_{on}1$s for YY_{DN} and FF_{DN} were twice as fast as for AA_{DN} and tSH2, while the $k_{off}1$s were half that of AA_{DN} and tSH2. The second set of (weaker) binding constants for YY_{DN} and FF_{DN} showed 10-fold higher K_D2s, with doubled on-rates and ~20-fold increased off-rates compared to their K_D1s. These data suggest that FF associates less efficiently with pCD3γ_{CD} because its fraction of weak binders is higher than YY's. Together with the HDX–MS findings, these data suggest that the closed conformation causes weaker binding. We tested the effects of ZAP-70 phosphorylation on pITAM-binding. Only phosphorylated pYY_{DN} had to be fitted with a different binding model (1:1) than its unphosphorylated form, consistent with a complete transition into an open high affinity conformation. The fact that the ratio of K_D1 and K_D2 is preserved for pFF_{DN} suggests the equilibrium is defined by the intra-molecular interactions of Y315/Y319, which are not altered by phosphorylation in the FF mutant. In addition, pYY_{DN} showed an ~1.5-fold decrease in $k_{on}2$, resulting in a higher K_D2. This suggests that other ZAP-70 phosphorylation sites can weaken pITAM-binding.

Our data demonstrate that pITAM-binding dynamics of ZAP-70 are determined by the weak binding of the closed conformation and strong binding of the open conformation, as well as their equilibrium. The closed conformation is more likely to associate with pITAMs. The propensity of the kinases to be in the closed conformation (FF_{DN} > YY_{DN}) correlates with increased dissociation rates. Stabilization of the open conformation by either phosphorylation or alanine mutation of Y315/Y319 strengthens the interaction of ZAP-70 with pITAMs and extends its TCR dwell-time. The latter increases the likelihood of ZAP-70 becoming activated by either CD4- or CD8-associated Lck, or another TCR associated ZAP-70.

ZAP-70 conformations determine dwell-time at TCR microclusters

ZAP-70 recruitment to microclusters (MCs) was analyzed to test if the different binding characteristics we observed in vitro exist *in vivo*. MCs are membrane structures that are formed around ligand-engaged TCR (Supplementary Fig. 5a)[35, 36]. MCs are crucial for signal initiation and contain most of the signaling molecules involved in early T cell activation. The interaction kinetics of wildtype ZAP-70 with the TCR were previously studied by fluorescence recovery after photo-bleaching using confocal microscopy ($FRAP^{Conf}$)[35]. ZAP-70 showed high exchange rates with the MCs during early (<5min) T cell activation and strongly reduced exchange rates at later stages (10–20min). Therefore, we analyzed early ZAP-70 recruitment to MCs by $FRAP^{Conf}$. GFP-tagged ZAP-70s (^{GFP}YY, ^{GFP}FF, GFPAA and ^{GFP}tSH2) or GFP-tagged TCR subunit CD3ζ (^{GFP}CD3ζ) were expressed in ZAP-70 deficient (P116) (Fig. 7a) and wildtype (E6.1) Jurkat T cell lines (Supplementary Fig. 5b). Cells were activated on antibody-coated glass surfaces to form MCs (Supplementary Fig. 5a) and the mobile fraction of MC-associated ZAP-70 or CD3ζ as well as their halftimes of recovery ($t_{1/2}$) were determined (Fig. 7b, Supplementary Fig 5c and Supplementary Table 3). It should be noted that binding to all ten pITAMs of the TCR/CD3-complex contributes to ZAP-70 clustering. Fluorescence recovery of ^{GFP}CD3ζ is minimal due to TCR immobilization on the antibody surfaces. Wildtype ZAP-70 (^{GFP}YY)

showed 80–90% mobile fraction and a $t_{1/2}$ of 19 seconds. ^{GFP}FF cannot compete with endogenous ZAP-70 for TCR binding and could only be analyzed in P116 T cells (Supplementary Fig 5a); ^{GFP}FF recovers completely (~100%) and has a $t_{1/2}$ of 14 seconds in P116 cells. The alanine mutant ^{GFP}AA only recovers to ~50% and has a slower $t_{1/2}$ of 28 seconds. ^{GFP}tSH2 showed similar values as ^{GFP}AA with ~60% recovery and $t_{1/2}$ of 26 seconds. Additionally, we analyzed ZAP-70 exchange rates in primary T cells from 5c.c7 TCR transgenic mice. These T cells recognize agonist peptide derived from moth cytochrome c (MCC; amino acid 88–103) presented by MHC class II molecules (specifically I-E^k). T cells were activated on I-E^k/MCC- and co-stimulatory CD80-coated glass surfaces. Smaller MCs and increased cytosolic background required exchange to be measured using total internal reflection microscopy (FRAP^{TIRF}). Mobile fractions and halftimes [^{GFP}YY (~80%; 11s), ^{GFP}FF (~75%; 3.5s), ^{GFP}AA (~45%; 9s) and ^{GFP}tSH2 (~60%; 11s)] showed similar results for the murine ZAP-70 mutants as was observed for human ZAP-70s in Jurkat T cells (Fig. 7c+d and Supplementary Table 3).

These results corroborate our *in vitro* binding studies; they show that the propensity of FF to be in the closed conformation causes high exchange rates and thus a low probability of activation. In contrast, the constitutively open AA is more stably associated with MCs and can initiate T cell activation. The intermediate mobile fraction of YY suggests that only a fraction of TCR-bound ZAP-70 becomes phosphorylated on Y315/319 at any given time and then binds similarly to AA. The fraction that does not encounter an active kinase is released like FF. The absent (FF) and reduced (AA) activity of the mutants in T cells suggests an optimized TCR dwell-time and phosphorylation sequence for ZAP-70 is required to achieve maximal kinase and cellular activity.

Discussion

We used a novel application of HDX–MS to show that TCR binding and/or ZAP-70 phosphorylation induces an open conformation by releasing interdomain B (I–B) and the kinase domain (KinD) from interdomain A (I-A). Release of the KinD from I-B requires phosphorylation of Y319. Mutant analysis indicates that tyrosine Y315 and Y319 are central in the formation of the closed form; while the phenylalanine (FF) mutant is more prone to assume the closed conformation, the alanine (AA) mutant is exclusively open. We utilized the Y315 and Y319 mutants to analyze the effects of distinct conformations on ZAP-70 catalytic activity and TCR binding. In contrast to the current general view, our data shows that the closed/auto-inhibited and open forms of ZAP-70 have similar potential to be activated by Lck as well as comparable maximal catalytic activity. Thus, the absence or reduction of T cell downstream signaling observed for FF and AA, respectively, cannot be explained through changes in intrinsic kinase activities. *In vitro* and *in vivo* binding studies show altered binding characteristics for the mutants. This suggests that ZAP-70 activity in T cells is controlled through its TCR dwell-time and subsequent association, which is stabilized by phosphorylation of Y315 and Y319. Additional post-translational modifications might contribute to the control of ZAP-70 recruitment and activation as well.

We propose a dynamic activation mechanism for ZAP-70 based on multiple conformational and receptor-binding equilibria (illustrated in Fig. 8). In quiescent T cells, ZAP-70 is

cytosolic and inactive, but with optimal TCR binding potential. Upon TCR association ZAP-70 assumes an open conformation, but its propensity for the closed conformation results in short receptor dwell-times. Consequently, the probability of ZAP-70 phosphorylation and activation are low. T cell recognition of peptide-MHCs and the co-recruitment of co-receptor-bound Lck increases the probability of ZAP-70 phosphorylation. Lck co-recruitment itself is controlled by antigen dwell-times with the TCR and the fraction of co-receptors coupled with Lck[37]. Phosphorylation of Y315/Y319 promotes an open conformation independently of pITAM association and therefore stabilizes TCR binding; this increases the dwell-time of ZAP-70 at T cell microclusters and facilitates further phosphorylation and, thus, catalytic activity and downstream signaling.

This novel mechanism explains how T cell quiescence and sensitivity are attained. Previous reports have shown that background TCR phosphorylation in peripheral T cells, thymocytes and T cell lines leads to ZAP-70 recruitment without T cell activation[11, 38, 39]. Our data suggest that short TCR dwell-times of ZAP-70 maintain T cells in a quiescent state despite this background stimulation by minimizing the chances of ZAP-70 encountering active kinases. On the other hand, stabilization of TCR binding upon T cell activation results in a 'switch-like' response. Trans-autophosphorylation[13] increases the numbers of ZAP-70 stably bound to TCR clusters and amplifies the signal. This mechanism creates an activation window that prevents responses to weak or endogenous ligands and therefore, avoids hyper-activation and autoimmune responses. On the other hand this mechanism also allows recognition of a small number of antigenic ligands to initiate appropriate immune responses.

Described advantages of enzyme recruitment to receptors that lack intrinsic catalytic activity include variable assembly to form distinct complexes, separation of catalytic activity from substrates and control over local concentrations of signaling molecules. Here, we have shown that the dynamic and flexible nature of these interactions makes the recruitment event itself an essential component of the activation mechanism. We suggest that our findings have broad implications for cellular pathways that utilize SH2 domains[40]. These include pathways used for signal transduction, cytoskeletal regulation, transcription and protein-degradation. Moreover, paired SH2 domains, as studied here, can be found in many enzyme types, such as kinases (e.g. ZAP-70 and Syk), phosphatases (e.g. SHP-2), hydrolases (e.g. PLCγ) and transcription factors (e.g. Spt6). More generally, any protein interaction domain with specificity for protein-motifs, post-translational modifications, nucleic acids or lipids[41] has the potential to be a part of similar mechanisms. Taken together, we suggest that the control of enzymatic activities through recruitment dynamics and their modulation is likely to be a common mechanism in biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Elisabeth Komives, Innokentiy Masslenikov, Christine Beuck and Martha Harber for valuable discussions. We are thankful to Bryan E. Jones (Eli Lilly) for access to the HDX–MS platform. Jamie Williamson (The Scripps Research Institute) for use of the BLI instrument. Barbara Varsanofieva for her assistance with FRAP

data acquisition. We thank Sreekanth Chalasani, Martin Hetzer and Tony Hunter for critical reading of the manuscript. We thank the Nomis Foundation, the Waitt Foundation and the James B. Pendleton Charitable Trust for their support.

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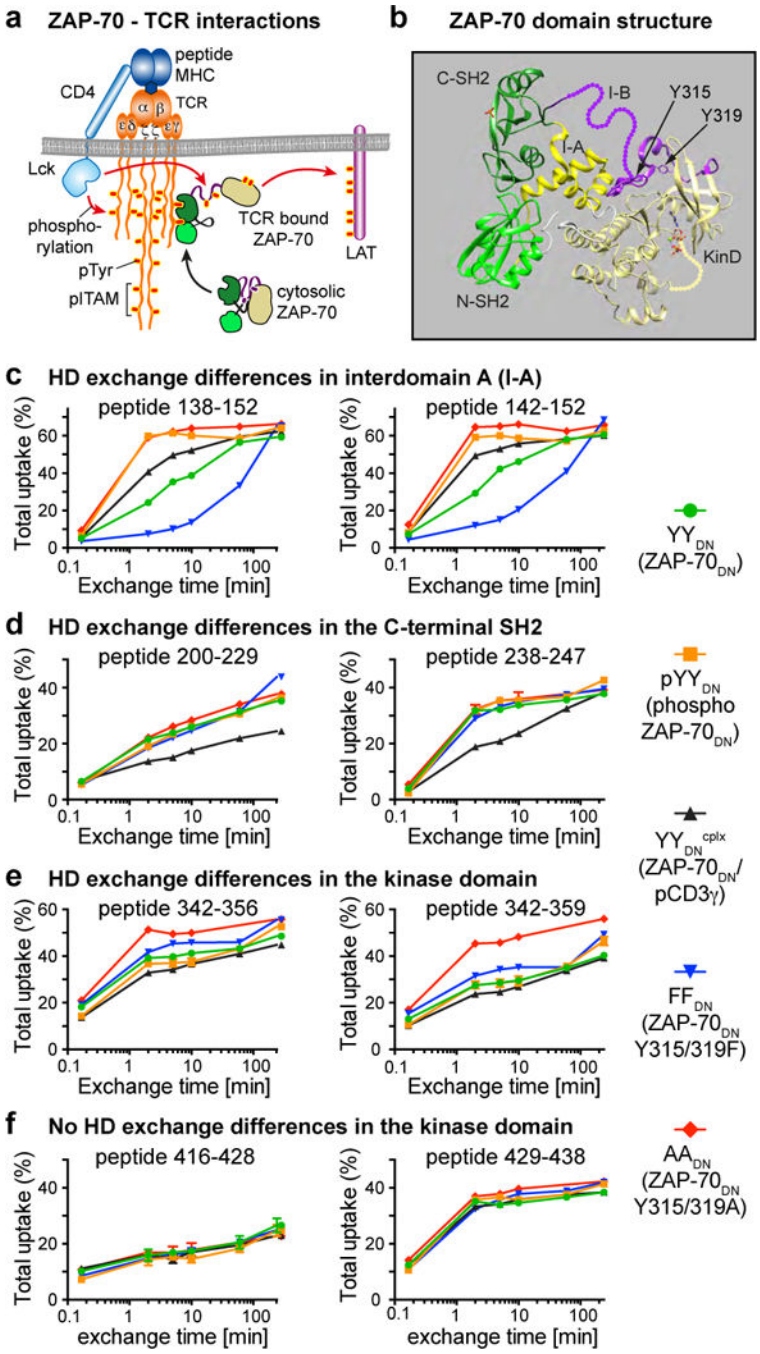

Fig. 1. ZAP-70 signaling, structure and HDX–MS

(**a**) Overview of antigen recognition and early TCR signaling. Red arrows indicate phosphorylation events. (**b**) ZAP-70 domain structure in the closed/auto-inhibited conformation[17]. Domains are indicated by different colors. Black arrows indicate positions of Y315 and Y319. Amino acids missing in the crystal structure are shown as an unstructured chain of dots. (**c**) Example of peptides in I-A with increased HDX upon phosphorylation, receptor binding or in the AA_{DN} mutant. Reduced exchanges are shown for FF_{DN}. (**d**) Example of peptides in tSH2 blocked by TCR binding. (**e and f**) Sample

peptides from the KinD show deuterium uptake differences upon phosphorylation, receptor binding or I-B mutations, whereas other peptides are not affected. DN abbreviates kinase dead. Each time point in c – f was generated by a single HDX–MS sample and is representative of three independent measurements.

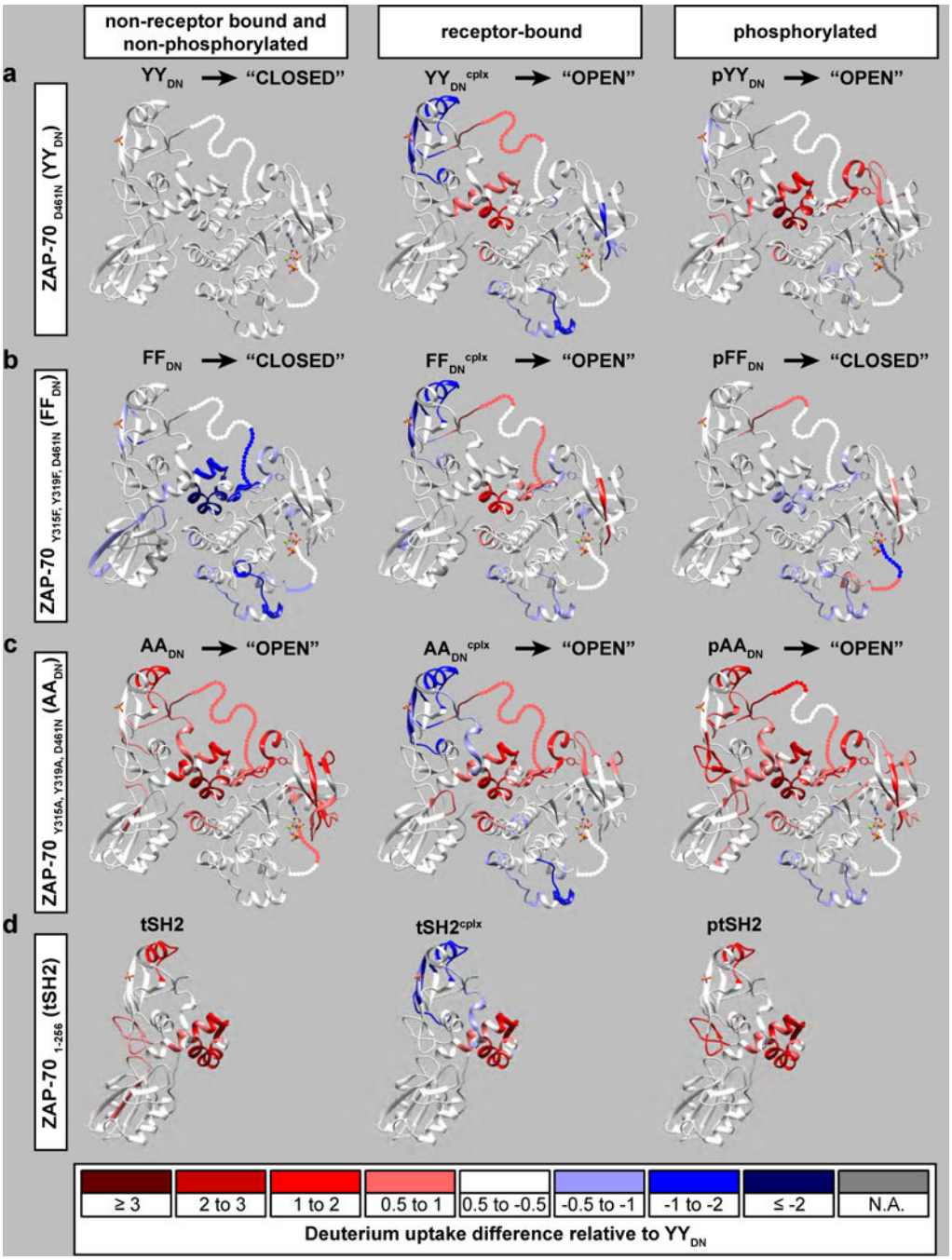

Fig. 2. HDX of ZAP-70 mutants superimposed on the closed/auto-inhibited structure

Regions with HDX for (**a**) YY_{DN}, (**b**) FF_{DN}, (**c**) AA_{DN} and (**d**) tSH2. Each ZAP-70 was analyzed in a non-receptor bound and non-phosphorylated (left), receptor bound (center), and phosphorylated state (right). Amino acid residues not covered by the structure[17] are shown as an unstructured chain of dots. Reduced or increased deuterium uptakes relative to YY_{DN} are color-coded (color bar, bottom). The resulting ZAP-70 conformations are indicated above each structure as "closed" (a.k.a. auto-inhibited) or "open". The data shown here was obtained from 2 minutes HD exchange reactions. The HD exchange was quantified

and assigned with data from ~150 distinct peptides per sample. The data represent three independent HDX-MS experiments per ZAP-70 form.

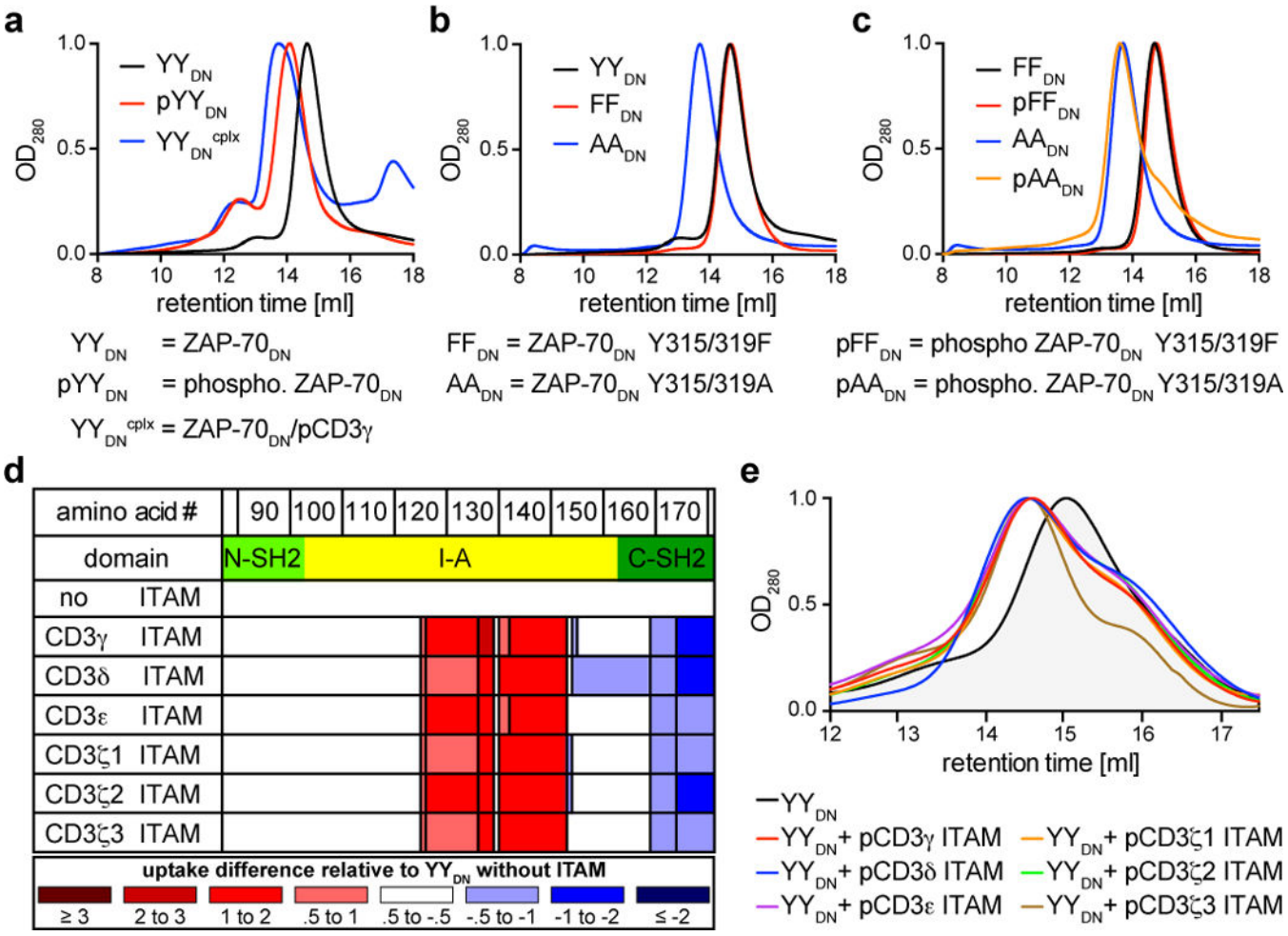

Fig. 3. Y315A/Y319A mutation or binding of any TCR pITAM opens ZAP-70

(**a**) Phosphorylation (pYY_{DN}) and TCR binding (YY_{DN}^{cplx}) reduce size exclusion chromatography (SEC) retention times of ZAP-70 (YY_{DN}). (**b**) Interdomain B (I-B) mutation of Y315 and Y319 to alanine (AA_{DN}) reduces the SEC retention time, whereas mutation to phenylalanine (FF_{DN}) has no effect. (**c**) Y315 and Y319 phosphorylation exclusively affect SEC retention times. (The size exclusion chromatography traces shown in (a – c) are representative of all purifications of the corresponding ZAP-70 forms.) (**d**) HDX–MS analyses of I-A from YY_{DN} in complex with phospho-peptides encoding all ITAMs of the TCR/CD3 complex shows opening of ZAP-70. Reduced (blue) or increased (red) deuterium uptakes relative to YY_{DN} are color-coded (bottom). Amino acid numbers are shown on top. (**e**) Reduced retention times of YY_{DN} in complex with all TCR pITAMs by analytical SEC.

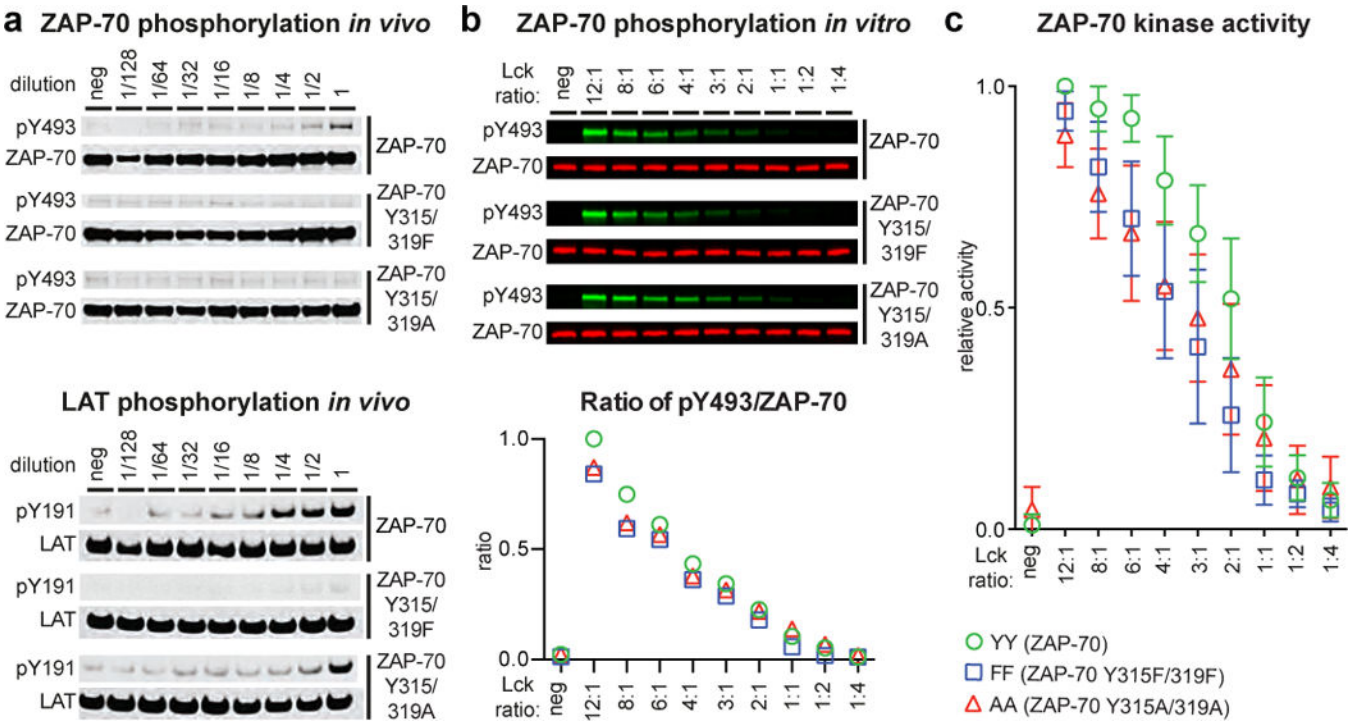

Fig. 4. Comparison of ZAP-70 kinase activities *in vivo* and *in vitro*

(**a**) Jurkat P116 expressing GFP–ZAP-70 fusion proteins were activated with serial dilutions of stimulating antibodies. Immunoblotting of ZAP-70 pY493 and total protein (top panel), and LAT pY191 and total protein (bottom panel). (**b**) Recombinant ZAP-70s were pre-activated with different ratios of Lck. Immunoblotting of ZAP-70 Y493 phosphorylation and total protein (top panel) and their ratios (bottom panel). (**c**) Normalized catalytic activities of pre-activated ZAP-70's from (b) analyzed by *in vitro* kinase assay using the recombinant cytosolic domain of LAT as substrate. Each data point in the kinase assay (c) represents the B_{max} value for fitted curves from the ELISA (duplicates of seven 2-fold dilutions for each kinase reaction) and the error bar represents the 95% CI. All data (a–c) is representative of three independent experiments.

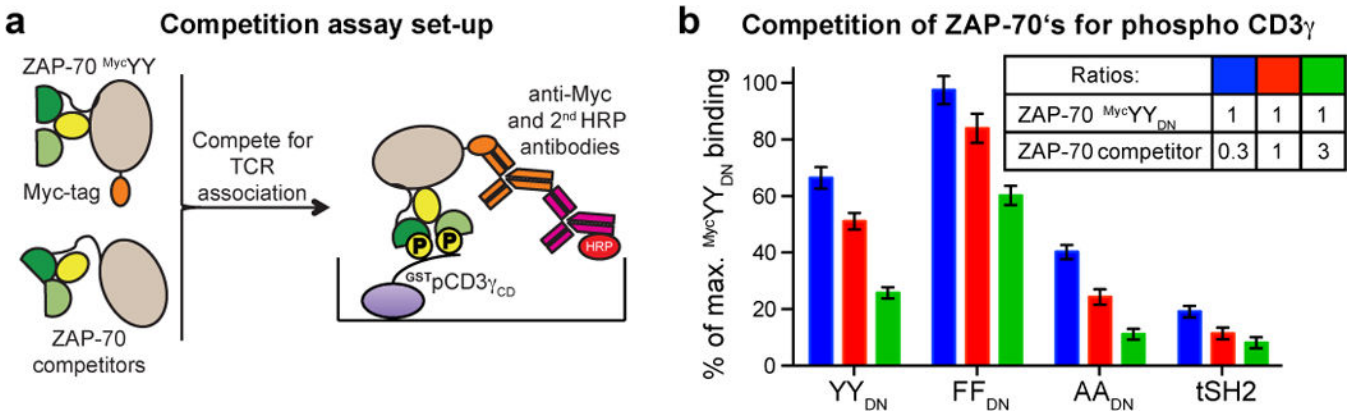

Fig. 5. Competition of different ZAP-70 forms for TCR binding

(**a**) Illustration of ELISA-based ZAP-70 competition assay using GST-pCD3γ_{CD} fusion protein as bait and $^{Myc}YY_{DN}$ for detection of binding. (**b**) Percentage of a constant concentration of $^{Myc}YY_{DN}$ binding to GST-pCD3γ_{CD} when competing with 3-fold less (blue), equimolar (red) or 3-fold excess (green) of untagged ZAP-70s (YY_{DN}, FF_{DN}, AA_{DN}, tSH2). Each data point was obtained from triplicate wells and the error bars represent s.e.m. The data is representative of 5 independent experiments.

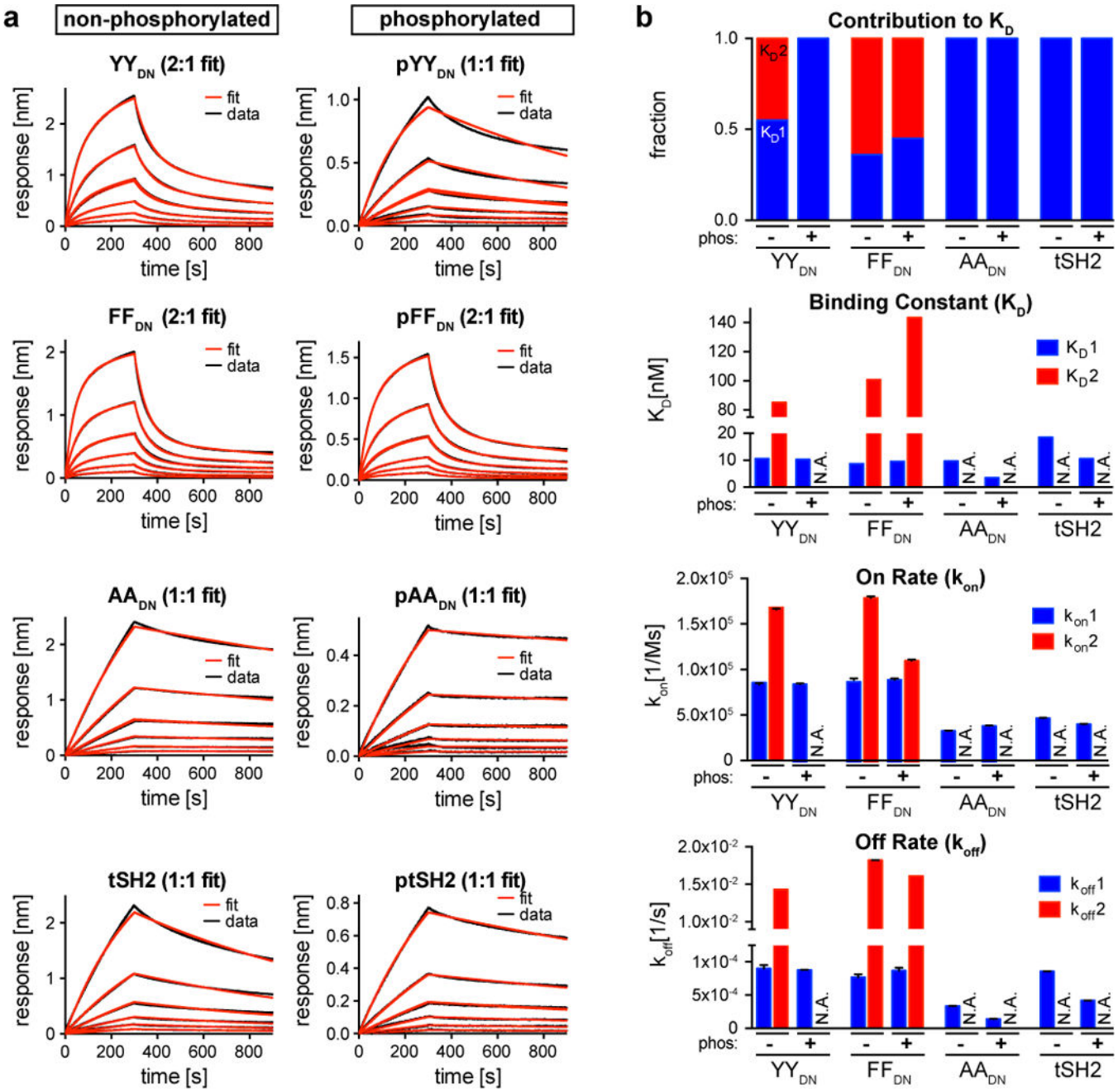

Fig. 6. Bio-layer interferometry (BLI) analyses of ZAP-70 binding to TCR

(**a**) Binding kinetics of ZAP-70's to immobilized pCD3γ_{CD} measured by bio-layer interferometry (BLI). The binding data (black) and fitted curves (red) for non- (left graphs) and phosphorylated (right graphs) ZAP-70s. Six different concentrations were measured and data fitted using either 1:1 or 2:1 binding models (indicated in brackets). (**b**) ZAP-70 fractions that contribute to the strong (K_D1, blue) or weak (K_D2, red) binding (top panel). Comparison of binding constants (K_D1 and 2), on-rates ($k_{on}1$ and 2), off-rates ($k_{off}1$ and 2) (bottom three panel, respectively). Non-phosphorylated (−) and phosphorylated (+) ZAP-70s were compared. N.A. indicates that a second binding constant was not observed. Each data

curve in (a) represents a single BLI measurement. Binding constants in (b) were determined from triplicates and error bars represent 99% CI. For all data X^2 and R^2 tests were performed (see supplementary Table 2). The data shown here is representative of 5 independent experiments.

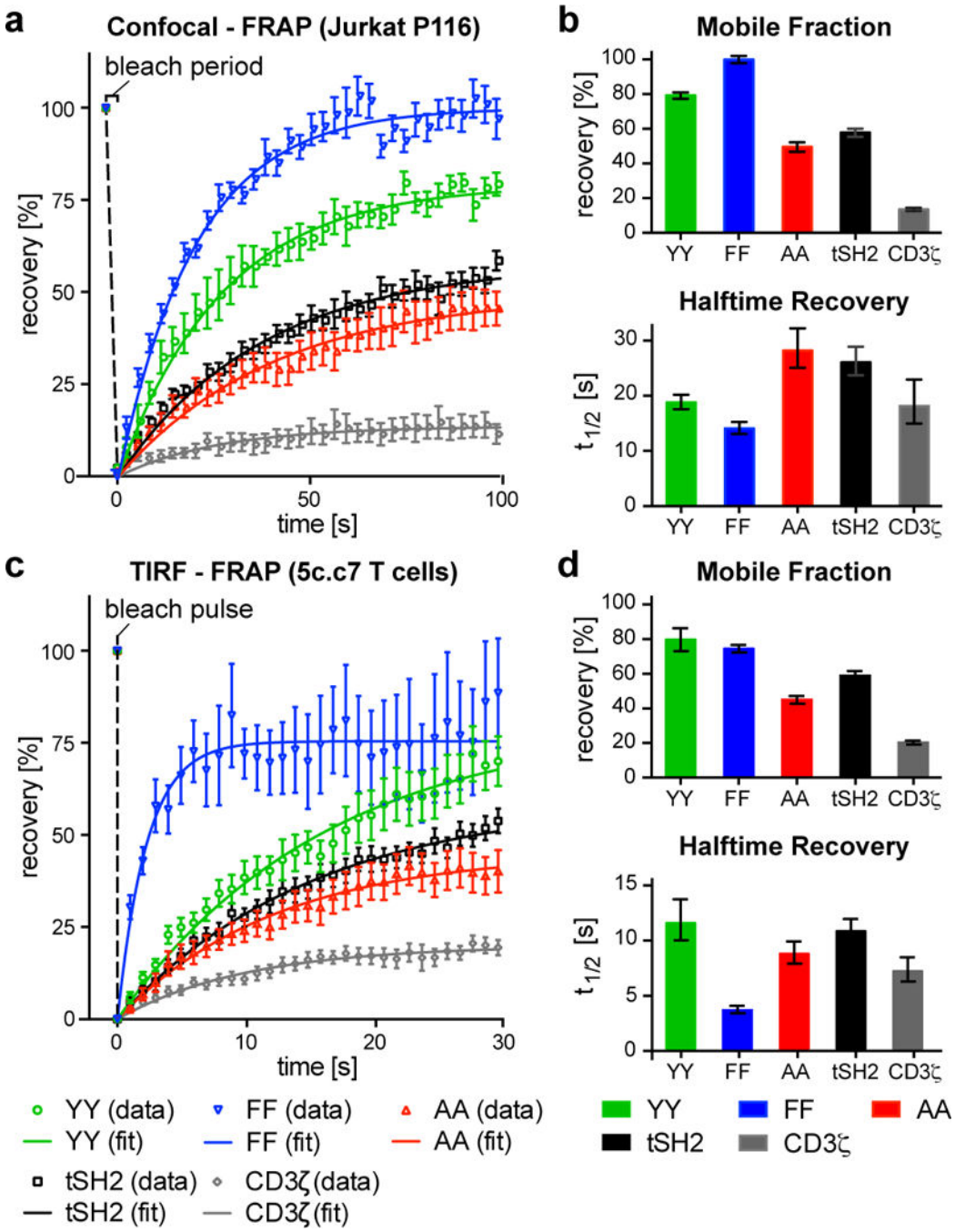

Fig. 7. Fluorescence Recovery After Photobleaching (FRAP) of ZAP-70 bound to TCR microclusters

(**a**) Jurkat P116 were transfected with fusion proteins (color coded index). T cells were activated on antibody-coated surfaces and $FRAP^{Conf}$ performed within 5min of surface binding. (**b**) Comparison of mobile fractions (top panel) and halftime recovery ($t_{1/2}$; bottom panel) in Jurkat P116. (**c**) Primary T cells from 5c.c7 transgenic mice infected with murine ZAP-70–GFP or CD3ζ–GFP fusion proteins were activated on immobilized I-E^k/MCC + CD80 surfaces. $FRAP^{TIRF}$ was performed within 5min of surface binding. (**d**) Comparison

of mobile fractions (top panel) and halftime recovery ($t_{1/2}$; bottom panel) in primary 5c.c7 T cells. FRAP data points (open symbols) and curves fitted to an exponential function in (a) and (c). Error bars represent s.e.m. [with (a) n_{YY}=8, n_{FF}=29, n_{AA}=7, n_{tSH2}=12 and $n_{CD3\zeta}$=7; and (c) n_{YY}=20, n_{FF}=22, n_{AA}=21, n_{tSH2}=24 and $n_{CD3\zeta}$=25]. Error bars for mobile fractions and $t_{1/2}$ in (b) and (d) represent the 95% CI. Data shown here represent 3 independent experiments.

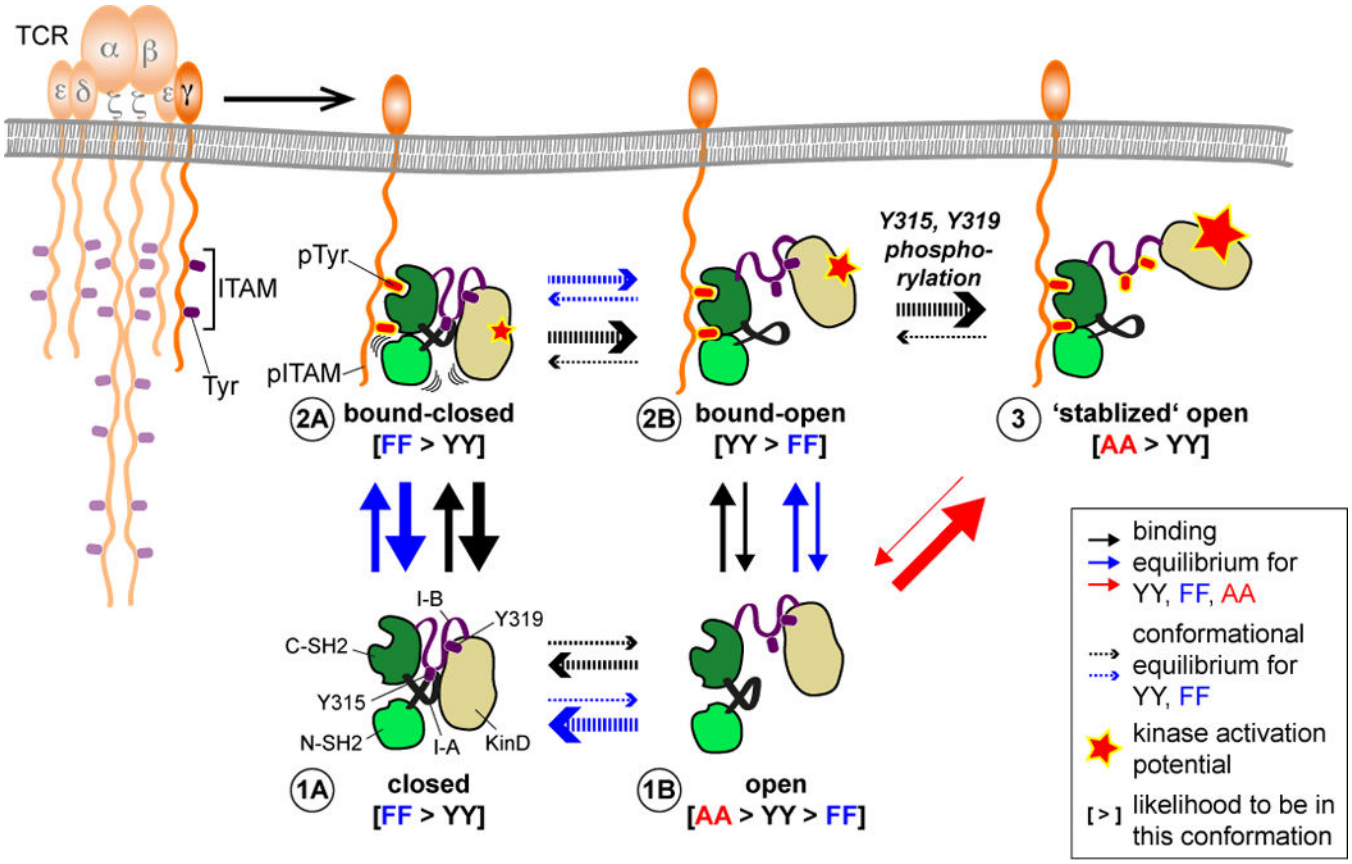

Fig. 8. Model: ZAP-70–TCR binding dynamics control its activation

The schematic shows binding (continuous arrows) and conformation equilibria (dotted arrows) for wildtype ZAP-70 (YY; black), and the Y315/Y319F (FF; blue) and Y315/319A (AA; red) mutants. Arrow thickness correlates with the rates of forward and backward reactions. The orders of probabilities for different ZAP-70s to exist in a specific state (circled numbers) are compared in brackets. In brief, cytosolic ZAP-70 is in a dynamic equilibrium between the closed/auto-inhibited (1A) and open conformations (1B). pITAM binding results in either a 'bound-closed' (2A) or bound-open (2B) complex. The 'bound-closed' complex is unstable, which commonly results in ZAP-70 release. Phosphorylation of Y315/Y319 'stabilizes' the open conformation and results in prolonged TCR association (3). ZAP-70 AA exists exclusively in an open conformation (1B) and always binds the TCR firmly (3). Once ZAP-70 binding to the TCR is stabilized, it is fully phosphorylated and acquires catalytic activity.